INTRODUCTION

Invasion and metastasis of malignantly transformed cells is a multistage process, which involves detachment of cells from the primary tumor, controlled degradation of structural barriers such as basement membrane and collagenous extracellular matrix (ECM), and migration of cells through degraded matrix (Stetler-Stevenson et al., 1993). Matrix metalloproteinases (MMPs) are a family of zinc-dependent neutral endopeptidases that are collectively capable of degrading essentially all ECM components, and they apparently play an important role in ECM degradation in tumor invasion and tumor-induced angiogenesis (Stetler-Stevenson et al., 1993). At present, 18 human members of the MMP gene family have been characterized, and according to their structure and substrate specificity they can be divided into subgroups of collagenases, gelatinases, stromelysins, membrane-type MMPs and other MMPs (Birkedal-Hansen et al., 1993; Kähäri and Saarialho-Kere, 1999). As fibrillar collagens are the most abundant structural components of the human connective tissues, the ability to degrade them is crucial for invasion and metastasis of neoplastic cells. Members of the collagenase subgroup of MMPs, i.e. collagenase-1 (MMP-1), collagenase-2 (MMP-8) and collagenase-3 (MMP-13), are the principal neutral proteinases capable of degrading native fibrillar collagens in the extracellular space. They all cleave type I, II and III collagens at a specific site generating 3/4 N-terminal and 1/4 C-terminal fragments, which rapidly denature at physiological temperatures and become susceptible to degradation by other MMPs e.g. gelatinases (see Birkedal-Hansen et al., 1993; Kähäri and Saarialho-Kere, 1997). MMP-13 also cleaves type II collagen.

Expression of collagenase-3 (MMP-13) and collagenase-1 (MMP-1) by transformed keratinocytes is dependent on the activity of p38 mitogen-activated protein kinase

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SUMMARY

Collagenase-3 (MMP-13) is a human matrix metalloproteinase specifically expressed by transformed squamous epithelial cells, i.e. squamous cell carcinoma (SCC) cells in culture and in vivo. Here, we have elucidated the signaling pathways regulating MMP-13 expression in transformed human epidermal keratinocytes, i.e. ras-transformed HaCaT cell line A-5 and cutaneous SCC cell line (UT-SCC-7). Treatment with tumor necrosis factor-α (TNF-α) resulted in activation of extracellular signal-regulated kinase (ERK)1,2, Jun N-terminal kinase and p38 mitogen-activated protein kinase (MAPK) in both cell lines. In addition, transforming growth factor-β (TGF-β) activated p38 MAPK in both cell lines, and ERK2 in A-5 cells. Selective inhibition of p38 activity with SB 203580 abolished the enhancement of MMP-13, as well as collagenase-1 (MMP-1) and 92-kDa gelatinase (MMP-9) expression by TNF-α and TGF-β. Blocking the ERK1,2 pathway by PD 98059 had no effect on the induction of MMP-13 by TNF-α or TGF-β, but potently suppressed MMP-1 and MMP-9 production. Inhibition of p38 activity by SB 203580 also suppressed collagenolytic activity produced by both cell lines and inhibited invasion of TNF-α or TGF-β stimulated A-5 cells through type I collagen and reconstituted basement membrane (Matrigel). These results show that activation of p38 MAPK pathway plays a crucial role in the invasive phenotype of transformed squamous epithelial cells, suggesting p38 MAPK as a target to specifically inhibit their invasion.

Key words: Matrix metalloproteinase, Collagenase, Invasion, MAPK, Squamous cell carcinoma

INTRODUCTION

Invasion and metastasis of malignantly transformed cells is a multistage process, which involves detachment of cells from the primary tumor, controlled degradation of structural barriers such as basement membrane and collagenous extracellular matrix (ECM), and migration of cells through degraded matrix (Stetler-Stevenson et al., 1993). Matrix metalloproteinases (MMPs) are a family of zinc-dependent neutral endopeptidases that are collectively capable of degrading essentially all ECM components, and they apparently play an important role in ECM degradation in tumor invasion and tumor-induced angiogenesis (Johnsen et al., 1998; Kähäri and Saarialho-Kere, 1999). At present, 18 human members of the MMP gene family have been characterized, and according to their structure and substrate specificity they can be divided into subgroups of
I collagen in N-terminal non-helical telopeptide region (Krane et al., 1996), and displays over 40-fold stronger gelatinase activity than MMP-1 and MMP-8 (Knäuper et al., 1996; Mitchell et al., 1996). In addition, MMP-13 cleaves type IV, X and XIV collagens, large tenasin C, fibronectin and aggrecan core protein (Knäuper et al., 1997; Fosang et al., 1996). Apparently due to its exceptionally wide substrate specificity, the expression of MMP-13 is limited to physiological situations in which rapid and effective remodeling of collagenous ECM is required, i.e. fetal bone development and postnatal bone remodeling (Johansson et al., 1997a; Stähle-Bäckdahl et al., 1997). On the other hand, MMP-13 obviously plays a crucial role in excessive degradation of collagenous ECM in osteoarthritic cartilage (Mitchell et al., 1996; Reboul et al., 1997; Wernicke et al., 1997), rheumatoid synovium (Lindy et al., 1997; Stähle-Bäckdahl et al., 1997; Wernicke et al., 1996), chronic cutaneous ulcers (Vaalamo et al., 1997), intestinal ulcerations (Vaalamo et al., 1998) and chronic periodontitis (Uitto et al., 1998).

The wide substrate specificity of MMP-13 clearly suggests that it is a potent proteolytic tool for invading neoplastic cells and, accordingly, the expression of MMP-13 has been detected in breast carcinomas (Freije et al., 1994; Heppner et al., 1996; Uría et al., 1997), squamous cell carcinomas (SCCs) of the head, neck and vulva (Johansson et al., 1997b, 1999; Airola et al., 1997; Cazorla et al., 1998), cutaneous basal cell carcinomas (Airola et al., 1997) and chondrosarcomas (Uría et al., 1998). In SCCs of the skin, oral cavity, pharynx, larynx and vulva, MMP-13 is expressed primarily by tumor cells at the invading edge of the tumor, and the expression of MMP-13 is especially abundant in SCCs showing local invasion or metastasis (Johansson et al., 1997b, 1999). In contrast, MMP-13 is not expressed by normal keratinocytes in intact or re-epithelializing epidermis, healthy oral mucosa or in culture, indicating that MMP-13 expression serves as a specific marker for transformation of keratinocytes (Johansson et al., 1997b,c, 1999; Vaalamo et al., 1997; Uitto et al., 1998). The expression of MMP-13 by cell lines established from SCCs of the head, neck and vulva, and by HaCaT cells, is enhanced by tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) (Johansson et al., 1997b,c, 1999), both of which may play a role in regulation of invasion capacity of SCCs in vivo.

In this study we have examined the role of mitogen-activated protein kinases (MAPKs) (Robinson and Cobb, 1997; Cohen, 1997) in the regulation of MMP-13 expression in transformed human epidermal keratinocyte-derived cell lines, i.e. ras-transformed HaCaT cell line (A-5) and cutaneous SCC cells (UT-SCC-7). We show that in both cell lines treatment with TNF-α or TGF-β results in activation of p38 MAPK, and that SB 203580, a specific inhibitor of p38 activity, blocks the enhancement of the expression of MMP-13, as well as that of MMP-1 and 92-kDa gelatinase (MMP-9) by TNF-α and TGF-β and inhibits invasion of A-5 cells through type I collagen and reconstituted basement membrane (Matrigel). These results provide evidence that the invasive phenotype of transformed squamous epithelial cells is dependent on p38 activity, suggesting that selective inhibition of the p38 MAPK pathway may serve as a target to inhibit invasion and metastasis of malignant epithelial cells.

### MATERIALS AND METHODS

#### Cell cultures

Ras-transformed HaCaT cell line A-5 (Boukamp et al., 1990) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS). Human SCC cell line UT-SCC-7, was established from metastasis of cutaneous SCC (Johansson et al., 1997b), was cultured in DMEM supplemented with 6 mM/L glutamine, nonessential amino acids and 10% FCS.

#### Reagents and antibodies

Human recombinant TNF-α and TGF-β1 were purchased from Sigma Chemical Co. (St Louis, MO, USA). Specific MEK1,2 inhibitor PD 98059 (2′-amino-3′-methoxyflavone) and p38 inhibitor SB 203580 ([4-(fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole]) were obtained from Calbiochem (San Diego, CA, USA). Phosphospecific ERK1,2, JNK/SAPK and p38 antibodies were purchased from New England Biolabs (Beverly, MA, USA).

#### RNA analysis

Total RNA was isolated from cells using a modified single-step phenol/chloroform extraction (Chomczynski and Sacchi, 1987). Portions of total RNA (20 µg) were analyzed by northern blot hybridizations as described previously (Johansson et al., 1997c), using cDNA probes labeled with [α-32P]dCTP by random priming. For hybridizations, MMP-13 cDNA fragments covering the coding region and part of the 3′-untranslated region of the human MMP-13 cDNA, altogether 1.9 kb, were used (Johansson et al., 1997c). In addition, a 2.0 kb human MMP-1 cDNA (Goldberg et al., 1986), a 1.3 kb rat GAPDH cDNA (Fort et al., 1985), a human 0.4 kb human c-jun cDNA (Angel et al., 1988), a human 1.2 kb junB cDNA (Schütte et al., 1989) and a human 3.1 kb c-fos genomic fragment (obtained from Amersham Corporation) were used as probes. The 32P-labeled cDNA-mRNA hybrids were visualized by autoradiography and the mRNA levels quantitated by scanning densitometry and corrected for the levels of GAPDH transcripts in the same RNA samples.

#### Determination of MAPK activation

Cells were treated with TNF-α (20 ng/ml) or TGF-β1 (5 ng/ml) for different periods of time, lysed in 100 µl of sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 µM DTT), sonicated, heated at 95°C for 5 minutes, fractionated by 10% SDS-PAGE and transferred to Hybond ECL membrane (Amersham, UK). Western blotting was performed as previously described (Reunanen et al., 1998) with phosphospecific antibodies for extracellular signal-regulated kinase (ERK)1,2, Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 antibodies and visualized by an enhanced chemiluminescence (ECL) detection system (Amersham).

#### Assay of MMP-13 and MMP-1 production

Equal portions of the conditioned medium of cells were fractionated by 7.5% SDS-PAGE, transferred to Hybond ECL membrane, and the amounts of MMP-13 and MMP-1 determined by western blot analysis using polyclonal antibodies against human recombinant MMP-13 (Freije et al., 1994), diluted 1:1000, and human MMP-1 (kindly provided by Dr H. Birkedal-Hansen, NIDR, Bethesda, MD, USA), diluted 1:2000, followed by ECL detection of specifically bound primary antibodies.

#### Assay of collagenase activity

Samples of conditioned medium were incubated with 1 mM aminophenyl mercuric acetate (Sigma) for 5 minutes at room temperature to activate latent MMP-13 and MMP-1. The samples were subsequently incubated with 20000 dpm 14C-labeled type I collagen for 24 hours at 25°C (Uitto et al., 1981). Thereafter, samples were heated at 95°C for 10 minutes in SDS-sample buffer and the...
uncleaved collagen α1 and α2 chains and their specific cleavage fragments were separated by 7.5% SDS-PAGE, visualized by fluorography and quantitated by densitometry.

**Gelatinase zymography**

Samples of conditioned medium were fractionated on 7.5% SDS-PAGE containing 1 mg/ml gelatin (G-6269, Sigma) and 0.5 mg/ml 2-methoxy-2,4-diphenyl-3(2H)-furanone (Fluka 645989) (O’Grady et al., 1984). The gels were washed for 30 minutes in 50 mM Tris, 0.02% NaN₃ and 2.5% Triton X-100, pH 7.5, and for another 30 minutes in the same buffer supplemented with 5 mM CaCl₂ and 1 mM ZnCl₂ (Heussen and Dowdle, 1980). The gels were then incubated in 50 mM Tris, 0.02% NaN₃, 5 mM CaCl₂ and 1 mM ZnCl₂ for 24 hours at 37°C, fixed in 50% methanol and 7% acetic acid, stained with 0.2% Coomassie Blue G250 (Kodak) and photographed.

**RESULTS**

**Invasion assays**

The invasion assays were performed as previously described (Albini et al., 1987). Cell culture inserts (Falcon 3097, Becton Dickinson, Franklin Lakes, NJ, USA) with 8.0 µm pore size were precoated with either 50 µg of reconstituted basement membrane (Matrigel, Becton Dickinson) or with a 1 mm thick type I collagen (Cellon Bovine Dermal Collagen, Cellon, Strassen, France) gel prepared by mixing eight volumes of collagen solution with one volume of 10× DMEM, and one volume of 0.2 M Hepes/0.04 M NaOH, pH 7.3, and allowed to polymerize at 37°C for 40 minutes. For invasion assays, cells (2×10⁵/chamber) suspended in DMEM containing 0.1% BSA were placed on top of the gel in the upper chamber in a final volume of 200 µl, with DMEM (700 µl) containing 10% FCS as chemoattractant in the lower chamber. After 24 hours (Matrigel) or 48 hours (type I collagen), cells on the upper surface were gently removed with a cotton bud and the invaded cells on the lower surface were fixed in 2% paraformaldehyde, counterstained with 0.1% Crystal Violet, and counted.

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**RESULTS**

**TNF-α activates ERK1,2, JNK and p38 MAPK in transformed keratinocytes**

Our recent observations show that MMP-13 is expressed by tumor cells in SCCs of the head, neck and vulva, as well as by cell lines established from corresponding tumors (Johansson et al., 1997b, 1999). In contrast, MMP-13 is not expressed by normal human epidermal keratinocytes in culture, in intact skin, or in re-epithelializing cutaneous wounds, indicating that expression of MMP-13 is a specific marker for transformation of keratinocytes (Johansson et al., 1997b, c; Vaalamo et al., 1997). The expression of collagenase-3 (MMP-13) by cell lines from SCCs of the head, neck and vulva is enhanced by TNF-α and TGF-β both of which may be present in the peritumoral environment in SCCs in vivo (Johansson et al., 1997b, 1999). To elucidate the signaling pathways regulating MMP-13 expression in transformed human epidermal keratinocytes we first examined activation of distinct MAPK pathways in A-5 cells, a ras-transformed HaCaT cell line (Boukamp et al., 1990), and UT-SCC-7 cells, a metastatic cutaneous SCC cell line (Johansson et al., 1997b). Initially, the cells were treated with TNF-α for different periods of time and the activation of ERK1,2, JNK/SAPK and p38 MAPK was determined by western blot analysis using antibodies specific for the phosphorylated, activated forms of these MAPKs. Treatment of A-5 and UT-SCC-7 cells with TNF-α resulted in rapid and transient activation of ERK2 (p42 MAPK), although in UT-SCC-7 cells ERK2 activation was only detected at 15 minutes of incubation (Fig. 1A,B). Very low levels of activated ERK1 were detected in both A-5 and UT-SCC-7 cells (Fig. 1A,B). Activation of JNK was also rapid and transient, the activated forms of JNK1 and JNK2 were first noted after 15 and 30 minutes of incubation in A-5 cells and at 15 minutes in UT-SCC-7 cells (Fig. 1A,B). Interestingly, treatment with TNF-α also resulted in marked and rapid (15 minutes) activation of p38 MAPK both in A-5 and UT-SCC-7 cells and the activation was rather persistent, as elevated levels of the phosphorylated form of p38 MAPK were still detected after 12 hours (Fig. 1A,B).

**TGFB-β activates p38 MAPK in transformed keratinocytes**

In the next set of experiments, we determined the effect of TGFB-β on the activation of distinct MAPK cascades in transformed keratinocytes. As shown in Fig. 2A, exposure of A-5 cells to TGFB-β (5 ng/ml) resulted in activation of ERK2, first noted at 1 hour and still detectable at 12 hours of incubation. In contrast, treatment of UT-SCC-7 cells with TGFB-β did not markedly activate ERK2 or ERK1 (Fig. 2B). TGFB-β also activated p38 MAPK in both A-5 and UT-SCC-7 cells, first detected after 1 hour. The activation of p38 was again sustained, as elevated levels of phosphorylated p38 were still detected after 12 hours in A-5 cells and 6 hours in UT-SCC-7 cells.

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**Fig. 1.** TNF-α activates ERK2, JNK and p38 MAPK in transformed keratinocytes. (A) Ras-transformed HaCaT cells (A-5) and (B) metastatic human skin squamous cell carcinoma cells (UT-SCC-7) were incubated with TNF-α (20 ng/ml) in serum-free medium for different periods of time as indicated. Activation of ERK1,2, JNK and p38 was determined by western blot analysis using phosphospecific antibodies against activated ERK1,2 (p-ERK1, p-ERK2), JNK (p-JNK1, p-JNK2) or p38 (p-p38). Representative western blots of three experiments are shown.
TNF-\(\alpha\), we examined the role of p38 and ERK1,2 MAPKs in the activation of MMP-13 production by TNF-\(\alpha\). In A-5 cells, expression of MMP-13 was slightly (2.3-fold) stimulated production of 72-kDa gelatinase (MMP-2) by A-5 cells and this upregulation was entirely inhibited by SB 203580 (Fig. 4). TGF-\(\beta\) strongly (2.3-fold) stimulated production of 72-kDa gelatinase (MMP-2) by A-5 cells and this upregulation was inhibited by SB 203580 and PD 98059 (Fig. 4).

**Distinct role of p38 MAPK and ERK1,2 in activation of c-jun, junB and c-fos expression by TNF-\(\alpha\) and TGF-\(\beta\)**

It has been shown that the enhancement of human MMP-13 gene transcription by interleukin-1 (IL-1), TNF-\(\alpha\), TGF-\(\beta\) and phorbol ester involves the AP-1 element located at -44 to -50 in the 5'-flanking region of human MMP-13 promoter (Péndas et al., 1997; Uria et al., 1997, 1998). The expression of the main components of the AP-1 dimers, c-Jun and c-Fos, is induced by distinct MAPKs in response to various extracellular stimuli, e.g. mitogens, cytokines and cellular stress (see Westermarck and Kähäri, 1999). In this context we also examined the role of p38 and ERK1,2 MAPKs in the activation of distinct components of the AP-1 complex. As shown in Fig.
Fig. 3. MMP-13 and MMP-1 expression in transformed keratinocytes is dependent on p38 MAPK activity. (A-C) A-5 cells were incubated for 24 hours in serum-free medium with (+) or without (−) TNF-α (20 ng/ml) or TGF-β1 (5 ng/ml) alone or in combination with p38 inhibitor SB 203580 (20 μM) or MEK1,2 activator inhibitor PD 98059 (40 μM) added 1 hour prior to TNF-α or TGF-β. (A) The mRNA levels for MMP-13, MMP-1 and GAPDH were determined by northern blot hybridizations. (B) proMMP-13 and proMMP-1 levels in the conditioned medium were determined by western blot analysis using antibodies against human MMP-13 and MMP-1. The levels of proMMP-13 and proMMP-1 were quantitated by densitometric scanning and are shown below the western blots relative to levels in untreated control cells (taken as 1.0). Migration positions of molecular mass markers (in kDa) are shown on the left. (C) Collagenolytic activity in the conditioned medium was determined after activation by APMA using 14C-labeled type I collagen as substrate. Cleaved α1 and α2 chains and the corresponding N-terminal 3/4 cleavage fragments α1A and α2A were separated by SDS-PAGE and visualized by fluorography. (D) A-5 cells were treated for 15 minutes with TNF-α (20 ng/ml) alone or with PD 98059 (40 μM), added 1 hour before. The activation of ERK1,2, JNK/SAPK and p38 was determined by western blot analysis using phospho-specific antibodies against activated ERK1,2 (p-ERK1, p-ERK2), JNK (p-JNK1, p-JNK2) or p38 (p-p38). (E) The levels of proMMP-13 and proMMP-1 in the conditioned medium of UT-SCC-7 cells treated as in A, were determined by western blot analysis using antibodies against human MMP-13 and MMP-1, as in B. The levels of proMMP-13 and proMMP-1 were quantitated by densitometric scanning and are shown below the western blots relative to levels in untreated control cells (taken as 1.0). Migration positions of molecular mass markers (in kDa) are shown on the left.

5A, treatment of A-5 cells with TNF-α results in the activation of c-jun, junB and c-fos mRNAs after 30 minutes and 1 hour of incubation. Interestingly, pre-treatment of A-5 cells with SB 203580 potently reduced the induction of junB mRNA abundance by TNF-α (Fig. 5A). In contrast, SB 203580 did not markedly alter induction of c-jun or c-fos mRNA levels, indicating that p38 MAPK is not essential for the activation of the expression of these AP-1 components by TNF-α (Fig. 5A). In parallel, PD 98059 entirely prevented the induction of c-fos mRNAs by TNF-α, and slightly reduced expression of junB mRNA (Fig. 5A).

Treatment of A-5 cells with TGF-β also resulted in activation of c-jun, junB and c-fos mRNAs, noted at 1 and 2 hours of incubation (Fig. 5B). Induction of c-jun and junB mRNAs was slightly reduced by SB 203580, whereas induction of c-fos expression was not altered by SB 203580 (Fig. 5B). In comparison, treatment of A-5 cells with PD 98059 prevented the induction of c-fos mRNAs, whereas the
inhibition of c-jun and junB mRNA induction was less potent (Fig. 5B).

Activity of p38 and ERK1,2 is required for invasion of transformed keratinocytes

Given the crucial role of p38 MAPK in the regulation of MMP-13, MMP-1 and MMP-9 expression we also examined the role of the p38 pathway in invasion capacity of transformed epidermal keratinocytes using A-5 cells as a model. Treatment of A-5 cells with TNF-α and TGF-β markedly and significantly enhanced their invasion through type I collagen and this was entirely inhibited by SB 203580 (20 μM) (Table 1). However, A-5 cell invasion was also potently inhibited by PD 98059, most likely due to its ability to suppress production of MMP-1 in the presence of TNF-α and TGF-β (Table 1).

In parallel experiments, the ability of A-5 cells to invade through reconstituted basement membrane (Matrigel) was also stimulated by TNF-α and to a lesser extent by TGF-β (Table 1). By analogy with the ability of SB 203580 to abrogate induction of MMP-9 production, it also potently inhibited the invasion of TNF-α or TGF-β-stimulated A-5 cells through Matrigel (Table 1). Similarly, A-5 cell invasion through Matrigel was potently inhibited by PD 98059 (Table 1).

DISCUSSION

Mitogen activated protein kinases (MAPKs) play an important role in translating extracellular stimuli to intracellular molecular signals, which regulate cell growth, differentiation, survival and death (Robinson and Cobb, 1997; Cohen, 1997). To date, three mammalian cytoplasmic MAPK pathways have been characterized in detail. Of these, the ERK1,2 pathway (Raf/MEK1,2/ERK1,2) is especially activated by mitogenic growth factors and phorbol esters, whereas JNK/SAPK (MEKK1-4/MKK4,7/JNK1-3) and p38 (MAPKKK/ MKK3,6/p38α,β) pathways are mainly activated by inflammatory cytokines and stress stimuli, including UV light and osmotic and oxidative stress (see Robinson and Cobb, 1997; Cohen, 1997). Phosphorylation of the conserved threonine and tyrosine residues of MAPKs by their specific upstream dual-specificity kinases (MAPKKs) results in their activation and subsequent translocation to the nucleus. Active MAPKs phosphorylate and

Table 1. Invasion of A-5 cells through type I collagen and Matrigel is dependent on the activity of p38 MAPK and ERK1,2

<table>
<thead>
<tr>
<th>Type I collagen</th>
<th>Matrigel</th>
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<tbody>
<tr>
<td>Invaded cells</td>
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</tr>
<tr>
<td>Value</td>
<td>Value</td>
</tr>
<tr>
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<td>11±17</td>
</tr>
<tr>
<td>TNF-α</td>
<td>4113±836</td>
</tr>
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<td>TNF-α + PD98059</td>
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<tr>
<td>TGF-β</td>
<td>492±1034</td>
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<td>26±24</td>
</tr>
<tr>
<td>TGF-β + PD98059</td>
<td>15±14</td>
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</tbody>
</table>

The invasion assays were performed as described in Materials and Methods using cell culture inserts precoated with either 1 mm thick type I collagen gel (Cellon Bovine Dermal Collagen) or 50 μg of reconstituted basement membrane (Matrigel). The number of invaded cells are expressed as mean ± s.d. (n=3).

Statistical analysis was performed using Student’s t-test. *Compared against untreated cells; ‡compared against corresponding TNF-α or TGF-β treated cells.

Fig. 4. Enhancement of 92-kDa gelatinase (MMP-9) expression in transformed keratinocytes by TNF-α and TGF-β is dependent on p38 MAPK and ERK1,2 activity. A-5 cells were incubated for 24 hours in serum-free medium with (+) or without (−) TNF-α (20 ng/ml) or TGF-β1 (5 ng/ml) alone or in combination with p38 inhibitor SB 203580 (20 μM) or MEK1,2 activator inhibitor PD 98059 (40 μM) added 1 hour prior to TNF-α or TGF-β. The levels of gelatinases in the conditioned medium were determined with gelatin zymography. The migration positions of 92-kDa and 72-kDa gelatinases are indicated. The levels of 92-kDa gelatinase and 72-kDa gelatinase were quantitated by densitometry and are shown below the zymogram relative to levels in untreated control cells (taken as 1.0).

Fig. 5. Distinct role of p38 MAPK and ERK1,2 in activation of the expression of c-jun, junB and c-fos mRNAs by TNF-α and TGF-β. A-5 cells were treated with (A) TNF-α (20 ng/ml) or (B) TGF-β1 (5 ng/ml) for different periods of time, alone or in combination with SB 203580 (20 μM) or PD 98059 (40 μM) as indicated, and the levels of c-jun, junB and c-fos and GAPDH mRNA were determined by northern blot hybridizations.

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Statistical analysis was performed using Student’s t-test. *Compared against untreated cells; ‡compared against corresponding TNF-α or TGF-β treated cells.
activate protein kinases, e.g. MAPK-activated protein kinases-1, -2 and -3, or transcription factors, e.g. Elk-1, c-Jun and ATF-2, which in turn induce expression of the components of the AP-1 complex (see Karin et al., 1997). There is considerable cross-talk between distinct MAPK cascades, as MEK1-1 have shown to activate MEK1,2, MKK4,7 and MKK3,6, and MKK4 can activate p38 MAPK (see Robinson and Cobb, 1997; Cohen, 1997). Evidence for the role of MAPKs in malignant transformation was initially provided by the finding that constant activation of ERK1,2 by constitutively active Raf-1 or MEK1 results in transformation of fibroblasts (Cowley et al., 1994; Mansour et al., 1994). Furthermore, recent studies have shown that the ERK1,2 pathway is activated in renal and breast carcinomas in vivo (Oka et al., 1995; Sivaraman et al., 1997), providing further evidence that constant activation of ERK1,2 is involved in malignant transformation of cells. However, the consequences of ERK1,2 activation are cell specific, as demonstrated by recent observation that constant activation of ERK1,2 cascade by Raf-1 can cause growth arrest in small cell lung carcinoma cells (Ravi et al., 1998).

In the present study we show for the first time that treatment of transformed human epidermal keratinocytes with TNF-α and TGF-β results in persistent activation of p38 MAPK and that specific inhibition of p38 activity by SB 203580 abrogates the upregulation of their collagenase-3 (MMP-13) and collagenase-1 (MMP-1) expression and collagenolytic capacity. Although TNF-α also activates ERK1,2 in these cells, selective blocking of the ERK1,2 pathway by MEK1,2 inhibitor PD 98059 had no effect on TNF-α-elicited activation of MMP-13 expression, indicating that activation of ERK1,2 is not essential for induction of MMP-13 expression by TNF-α in transformed keratinocytes. In comparison, TGF-β also activated p38 MAPK in both cell lines, although the activation occurred at a somewhat later time point than with TNF-α. The enhancement of MMP-13 expression by TGF-β was abrogated by SB 203580, indicating that it also requires activity of p38 MAPK. Interestingly, TGF-β did not activate JNK/SAPK in the cell lines examined, indicating that activation of the JNK/SAPK pathway is not essential for enhancement of MMP-13 expression in transformed keratinocytes. Furthermore, inhibition of the ERK1,2 cascade by PD 98059 had no effect on enhancement of MMP-13 expression by TGF-β in A-5 cells, and in UT-SCC-7 cells ERK1,2 was not even activated by TGF-β, indicating that in these tumorigenic transformed keratinocyte cell lines activation of ERK1,2 is not involved in the upregulation of MMP-13 expression. These results clearly show that activation of p38 MAPK is critical in activation of the collagenolytic capacity of transformed keratinocytes by TNF-α and TGF-β.

Our recent observations show that MMP-13 is specifically expressed by transformed keratinocytes in SCCs of the head, neck and vulva (Johansson et al., 1997b; 1999), but not in normal keratinocytes in culture or in vivo (Johansson et al., 1997b,c; Vaalamo et al., 1997). In a subset of SCCs, MMP-13 expression is also detected in stromal fibroblasts (Johansson et al., 1997b; 1999). Interestingly, human skin fibroblasts in culture express MMP-13 only when in contact with three-dimensional collagen and this induction requires p38 MAPK activity (Ravanti et al., 1999), providing further evidence for an important role of p38 MAPK in regulation of collagenolytic capacity of cells. In dermal fibroblasts, activation of MMP-1 and stromelysin-1 (MMP-3) expression by IL-1 is also mediated by p38 MAPK (Ridley et al., 1997). Furthermore, our recent observations show that activation of MMP-1 expression in dermal fibroblasts by lipid second messenger ceramide and tumor promoter okadaic acid is mediated by p38 MAPK, in addition to ERK1,2 and JNK/SAPK pathways (Reunanen et al., 1998; Westerman et al., 1998). In addition, the observations in the present study show that enhancement of MMP-9 production by TNF-α and TGF-β by A-5 cells is dependent on p38 activity, although it is also inhibited by blocking the ERK1,2 pathway. These results, together with previous observations (Simon et al., 1998), show that p38 MAPK activation is critical for induction of the expression of three invasion-associated MMPs, i.e. MMP-13, MMP-1 and MMP-9, by transformed keratinocytes.

Invasion of malignant cells is a multistep process in which cellular motility is coupled to proteolysis, and involves interactions with the extracellular matrix (Stetler-Stevenson et al., 1993). It is evident, that invasion of SCCs in vivo involves interplay between tumor cells, stromal cells and inflammatory cells, all of which express a distinct pattern of MMPs contributing to degradation of stromal ECM components (see Johnsen et al., 1998; Kähäri and Saarialho-Kere, 1999). However, degradation of fibrillar collagens by collagenolytic MMPs is essential for invasion of SCC cells, as fibrillar collagens of type I and III constitute the majority of the stromal ECM of SCCs. In this study, invasion of A-5 cells was potently inhibited by p38 MAPK inhibitor SB 203580, indicating that p38 activity is required for invasion capacity of transformed keratinocytes. Although invasion of tumor cells also requires enhanced cell motility, which may also be dependent on p38 MAPK activity (Rousseau et al., 1997), it is evident that cells cannot migrate through native type I collagen without degrading it first by MMP-13 or MMP-1. In addition, it has been shown that migration of epidermal keratinocytes on native type I collagen is dependent on their ability to degrade it with MMP-1 (Pilcher et al., 1997). Therefore, it is possible that the ability of SB 203580 to inhibit invasion of transformed keratinocytes through type I collagen gel is at least in part due to reduced production of MMP-13 and MMP-1. In addition, A-5 cell invasion through basement membrane (Matrigel) was entirely abrogated by SB 203580, indicating that the activity of p38 is required for invasion of these cells through basement membrane. Together these observations show that the p38 MAPK pathway plays an important role in invasion of transformed keratinocytes. However, invasion of A-5 cells through type I collagen and Matrigel was also potently inhibited by PD 98059, indicating that the ERK1,2 pathway also plays an important role in the overall invasion capacity of transformed keratinocytes.

The expression of MMP-13 in HaCaT and SCC cells is potently enhanced by TNF-α and TGF-β (Johansson et al., 1997b,c, 1999) and the activation of human MMP-13 promoter by phorbol ester, IL-1, TNF-α and TGF-β involves an AP-1 binding element in the promoter region (Péndas et al., 1997; Uria et al., 1997, 1998). In A-5 and UT-SCC-7 cells, TNF-α and TGF-β rapidly induce the expression of the mRNAs for c-jun, junB, and c-fos. Inhibition of p38 activity by SB 203580 most potently inhibited induction of junB mRNA, whereas it did not markedly inhibit induction of c-jun or c-fos expression, suggesting a role for JunB containing AP-1 transcription factors in induction of the expression of MMP-13, MMP-1 and MMP-9. However, it should be noted that JunB is a weak activator of AP-1 driven promoters, as compared to c-Jun (Chiu et al., 1989;
Serve as a specific target to potently suppress invasion and p38 MAPK activity appears to essential for invasive phenotype of cells (Cowley et al., 1994; Mansour et al., 1994), whereas constant activation of ERK1,2 is sufficient for transformation. MMP expression. Our results also provide evidence that inhibition of the activity together with recent observations (Gum et al., 1997; Ridley et al., 1998), indicate that inhibition of the activity to MMP-13, MMP-1 and MMP-9 expression also involves stabilization of the corresponding transcripts. 

SCCs of the head and neck are malignant tumors with a high capacity for invasion and metastasis in the early stage of tumor development, resulting in poor prognosis. As MMPs have been implicated in growth and invasion of SCCs (see Johnsen et al., 1998; Kähäri and Saarialho-Kere, 1999), inhibition of MMP activity appears to be a potent way of inhibiting tumor cell invasion. At present, several synthetic small molecule MMP inhibitors are in clinical trials to assess their ability to inhibit growth and invasion of various malignant tumors in vivo (Brown, 1998; Kähäri and Saarialho-Kere, 1999). In addition, we have shown that adenovirus-mediated gene delivery of tissue inhibitor of metalloproteinases (TIMP)-1, -2 and -3 into malignant melanoma cells is an effective way of inhibiting their invasion (Ahonen et al., 1998). The results of the present study, together with recent observations (Gum et al., 1997; Ridley et al., 1997; Reunanen et al., 1998; Simon et al., 1998; Westermark et al., 1998), indicate that inhibition of the activity of distinct MAPKs may serve as a potent way of inhibiting MMP expression. Our results also provide evidence that ERK1,2 and p38 MAPKs play a distinct role in tumor biology: constant activation of ERK1,2 is sufficient for transformation of cells (Cowley et al., 1994; Mansour et al., 1994), whereas p38 MAPK activity appears to essential for invasive phenotype of transformed epithelial cells. Together these observations suggest that selective inhibition of the p38 MAPK cascade may serve as a specific target to potently suppress invasion and metastasis capacity of neoplastic cells in vivo.

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